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Adjuvants and immunostimulants in fish vaccines: Current knowledge and future perspectives

Carolina Tafalla[1], Jarl Bøgwald[2] and Roy A. Dalmo[2*]

[1]Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Madrid, Spain.

[2]Norwegian College of Fishery Science, University of Tromsø, N-9037 Tromsø, Norway.

*Corresponding authors: Roy A. Dalmo. Address: Norwegian College of Fishery Science, University of Tromsø, N-9037 Tromsø, Norway. Tel.: +47 77644482; Fax: +47 77646020; Email: roy.dalmo@uit.no; Carolina Tafalla. Address: Centro de Investigación en Sanidad Animal (CISA-INIA). Carretera de Algete a El Casar km. 8.1. Valdeolmos 28130 (Madrid). Spain. Tel.: 34 91 6202300; Fax: 34 91 6202247; Email: tafalla@inia.es.

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Abstract

Vaccination is the most adequate method to control infectious diseases that threaten the aquaculture industry worldwide. Unfortunately, vaccines are usually not able to confer protection on their own; especially those vaccines based on recombinant antigens or inactivated pathogens. Therefore, the use of adjuvants or immunostimulants is often necessary to increase the vaccine efficacy. Traditional adjuvants such as mineral oils are routinely used in different commercial bacterial vaccines available for fish; however, important side effects may occur with this type of adjuvants. A search for alternative molecules or certain combinations of them as adjuvants is desirable in order to increase animal welfare without reducing protection levels. Especially, combinations that may target specific cell responses and thus a specific pathogen, with no or minor side effects, should be explored. Despite this, the oil adjuvants currently used are quite friendlier with respect to side effects compared with the oil adjuvants previously used. The great lack of fish antiviral vaccines also evidences the importance of identifying optimal combinations of a vaccination strategy with the use of a targeting adjuvant, especially for the promising fish antiviral DNA vaccines. In this review, we summarise previous studies performed with both traditional adjuvants as well as the most promising new generation adjuvants such as ligands for Toll receptors or different cytokines, focusing mostly on their protective efficacies, and also on what is known concerning their effects on the fish immune system when delivered *in vivo*.

Contents

1. Introduction

Disease prevention by vaccination is, on economic, environmental and ethical grounds the most appropriate method for pathogen control currently available to the aquaculture sector. Traditionally, vaccines comprise either live-attenuated, replicating pathogens or non-replicating, inactivated pathogens or their subunits. In many countries, live vaccines are not approved for use in aquaculture for safety reasons, while inactivated vaccines based on either killed pathogens or isolated non-replicating pathogen subunits, are in many cases, weakly immunogenic. Thus, adjuvants or immunopotentiators, are highly required for the elicitation of immune responses that may be 100% protective against certain pathogens.

During the past, fish vaccines were made by a trial-and-error approach (conventional vaccine design) including pathogen identification, pathogen cultivation, and vaccine formulation containing whole cell preparation and oils. Through this strategy, vaccines based on whole inactivated extracellular bacterial pathogens were quite efficient; resulting in important reductions in mortalities and antibiotic usage in the aquaculture industry [1]. However, many of the economically disastrous diseases of today are due to intracellular pathogens, and for this type of pathogens the production of effective vaccines has not been an easy task. In this sense, the most promising future vaccines that induce protection against viruses are DNA vaccines. Intramuscular injection of a DNA plasmid encoding an immunogenic antigen has proved very effective in fish, in comparison to the results obtained in other animal models such as mammals [2]. Because the antigen is produced by the fish cells, it is exposed on the cell surface both directly or processed in the context of both major histocompatibility complex (MHC) class I and MHC class II, thus effectively triggering both humoral and

cellular immune responses. Although DNA vaccines offer a number of advantages over conventional vaccines, there are still many aspects that may be optimised with adjuvant help such as alternative routes of immunisation that allow mass-vaccination. Therefore, fish vaccine approaches must be subjected to rational vaccine design wherein a combination of a tailored adjuvant system with the most appropriate antigen is used to create vaccines that may provide a more effective immune response against a specific pathogen with minimal side effects.

On the other hand, many aspects of fish immunology are still unknown and we are far from close to understanding on which immune mechanisms the protection against many of these pathogens resides [3]. Moreover, as we know of today, there are close to 22000 different fish species, and most of them have their "immune peculiarities". Without a doubt the innate defence system of fish is strongly developed and may cope with many infectious agents, helping the fish to eradicate viruses, bacteria and even parasites. However, many infectious agents resist innate defence mechanisms, and then an adaptive immune response, present for the first time in evolution in teleost fish, must come into play to fight these pathogens, being this adaptive response the basis for vaccinology. The adaptive immune response provides the vertebrate immune system with the ability to recognise and remember specific pathogens, to be able to mount stronger and faster responses each time this pathogen is encountered. In higher vertebrates, adaptive immunity to extracellular pathogens is generally mediated by humoral immune responses (antibodies), while immunity to intracellular pathogens (including viruses) often relies on cellular immune responses (cytotoxic T cells). In fish, and despite the fact that the main elements for an adaptive immune response are present in most species, the regulation of these elements greatly differs from mammalian systems and even among different species. Both immunoglobulin (Ig) or B cell receptor

(BCR) and T cell receptor (TCR) genes are known among all lineages of gnathostomes (jawed vertebrates), but fish Ig are expressed as only as three isotypes (IgM, IgD and IgT) with no isotype switching and low affinity maturation [4]. Interestingly, there is a tight link between the innate and adaptive system that has not been much explored in fish immunology. This link, governed by several innate receptors and signalling molecules such as cytokines and transcription factors, may provide the key for the future rational design of vaccine adjuvants, since recent advances in immunology have shown that the magnitude and specificity of the signals perceived by the innate immune cells following vaccination shape subsequent adaptive immune responses [5].

2. Principles of adjuvant actions

Adjuvants (from Latin *adjuvare* meaning "to help") have traditionally been defined as helper substances that increase the magnitude of an adaptive response to a vaccine (potency), or ability to prevent infection and death (efficacy). But nowadays scientists have acknowledged that adjuvants may become more important in the way adjuvants guide the type of adaptive response against a specific pathogen. Therefore, adjuvants have been now defined as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen [6]. They can be classed according to their chemical nature or physical properties, however since even related compounds can have very different immunomodulating capacities, novel classifications have focused on the immunological events they induce, even though for many of them the exact mechanism of action is unknown. At present, the classification of adjuvants that distinguishes between signal 1 facilitators and signal 2 facilitators has been widely accepted [7]. According to this two-signal model, both the presentation of an antigen

148 (signal 1) and the additional secondary signals (signal 2) are required for activation of
149 specific T and B lymphocytes, which form the adaptive arm of the immune system [8].
150 The signal 1 facilitators influence the fate of the vaccine antigen in time, place, and
151 concentration, ultimately improving its immune-availability, while signal 2 facilitators
152 provide the co-stimulation signals during antigen recognition that will provide an
153 adequate environment for the most adequate antigen-specific immune response.

154 Another important aspect of the immune response against many adjuvants is the
155 recognition of microbes through the detection of conserved molecular patterns,
156 designated as pathogen-associated microbial patterns (PAMPs), through pathogen
157 recognition receptors (PRRs) that include Toll-like receptors (TLRs), NOD-like
158 receptors, dectin-1 or RIG-like helicases which are predominantly found on cells of the
159 innate immune system. Nowadays, this recognition is considered critical in signal 2
160 induction and downstream activation of distinct T helper cell subsets; however, other
161 authors make a distinction and refer to adjuvants that trigger PRRs as signal 0
162 adjuvants. In fact, recent work on adjuvants has especially focused on different PRR
163 ligands including different PAMPs, and as well as other endogenous TLR ligands
164 (Damage-associated molecular pattern molecules or DAMPs) such as heat-shock
165 proteins (hsp), studying their ability to induce targeted Th responses. Once there is a
166 production and expression of IL-2 (T cell growth factor) and its alpha-subunit of the IL-
167 2 receptors (CD25) during e.g. activation of naïve Th cells into Th0 cells, proliferation
168 of Th cells starts. Th0 cells will differentiate to Th1 or Th2 cells depending on the
169 cytokine environment, wherein IFN-γ drives Th1 cells while IL-4 induces Th2 cell
170 production/differentiation [9]. Additionally, after many cell generations, the Th cells
171 progenitors differentiate to effector Th cells, memory Th cells and regulatory Th cells.
172 Different vaccine adjuvants that are in use in veterinary and human medicine aid

differentiation of Th cells into several T cell lineages – such as Th1, Th2, Th9 and Th17 [10, 11]. In Table 1, we describe known adjuvant actions by both commercial and experimental adjuvants, used mainly in human medicine.

3. Signal 1 adjuvants used in fish vaccinology

3.1. Oil emulsions

To increase the immunogenicity of an antigen, a slow release is often achieved through the introduction of the antigen in the context of an emulsion. An emulsion is defined as a dispersion of a liquid, called the dispersed phase, in a second liquid, called the continuous phase in which the first one is not miscible. In vaccine formulations, these phases are water (antigenic media) and oil. In order to stabilise the emulsions, surfactants are added. A surfactant is a compound containing a polar group that is hydrophilic and a non-polar group that is hydrophobic and often composed of a fatty chain. Surfactants can be defined by their hydrophilic: lipohilic balance (HLB) value that gives information on their relative affinity for both phases. According to the HLB value of the surfactant, different kind of emulsions can be achieved [12]. Those having a low HLB value have a high affinity for oily phases and render W/O emulsions, whereas those with a high HLB value have a high affinity for the aqueous phase and render O/W emulsions, which are well tolerated but induce a shorter term immune response. With certain specific surfactant systems, when the HLB value is intermediate, W/O/W emulsions can be achieved. In this case, the continuous phase is aqueous and the dispersed phase is oil. But inside the oil droplets, an entrapped aqueous phase is found. This type of emulsions has shown to generate long-term immune responses with various antigens.

198

199 *3.1.1. Freund´s complete adjuvant*

200 The most widely used and most effective adjuvant for experimental purposes has
201 been Freund´s complete adjuvant (FCA). FCA is composed of heat-killed *Mycobacteria*
202 and a mineral oil with surfactant [13]. Before injection, the antigen in an aqueous
203 solution is mixed with the FCA producing a stable W/O emulsion. Immunisation with
204 FCA and antigens results in strong Th1 and Th17 responses predominantly via the
205 MyD88 pathway. Unfortunately, the use of FCA has been associated with a variety of
206 severe side effects including injection site granuloma; therefore, the use of FCA has
207 been limited to research on animals including fish for establishing an effective immune
208 response. Furthermore, the use of FCA in fish has not always resulted in an increase in
209 immunogenicity or protection.

210 Pasteurellosis, caused by *Pasteurella piscicida*, also named *Photobacterium*
211 *damsela* subsp. *piscicida* is one of the major diseases in many species of wild and
212 farmed fish in Asia, USA and Europe. In yellowtail (*Seriola quinqueradiata*), a
213 susceptible species, vaccination against pasteurellosis has been assayed with a
214 lipopolysaccharide (LPS)-mixed chloroform-killed bacterin which resulted in protection
215 against challenge with the virulent bacterium. In this case, the inclusion of FCA in the
216 vaccine did not significantly enhance the protective effect [14].

217 *Streptococcus iniae* is a Gram positive bacterium associated with disease in
218 several commercial species including tilapia (*Oreochromis aureus* and *O. niloticus*),
219 yellowtail, hybrid striped bass (*Morone saxatilis*), turbot (*Scophthalmus maximus*), and
220 rainbow trout (*Oncorhynchus mykiss*). Vaccination of rainbow trout with a formalin-
221 killed culture of *S. iniae* resulted in good protection against experimental challenge that
222 was not significantly potentiated in the presence of FCA [15].

223 *Aeromonas hydrophila* is a Gram-negative bacterium known to cause motile
224 aeromonas septicemia (MAS) in freshwater fish farming. The major adhesin of *A.*
225 *hydrophila*, a 43 kDa outer membrane protein, was cloned, expressed and emulsified in
226 FCA to be used in a vaccine for the blue gourami (*Trichogaster trichopterus*) [16]. The
227 vaccine was intraperitoneally (i.p.) injected and after three weeks a booster was given
228 without FCA. Two weeks after the booster, the fish were challenged with two strains of
229 *A. hydrophila*. The recombinant adhesin protected against challenge with both the
230 homologous strain of *A. hydrophila*, and the heterologous strain, providing the same
231 immune protection as the native adhesin [16].

232 *Aeromonas salmonicida* is the etiological agent for furunculosis. In a study in
233 coho salmon (*Oncorhynchus kisutch*), formalin-killed *A. salmonicida* was i.p. injected
234 in the absence or presence of FCA. In this model, the best protection was found for the
235 FCA adjuvanted vaccine. Interestingly, fish injected with FCA (without antigen) gave
236 some protection even 90 days after challenge [17]. Injection of inactivated *M. bovis* may
237 induce innate defence mechanisms that may result a certain degree of protection to a
238 heterologous pathogen, as shown by Kato *et al*. [18] where Japanese flounder
239 (*Paralichthys olivaceus*) were partially protected against nocardiosis with FCA. In a
240 recent study, Zheng *et al*. [19] compared naturally occurring adjuvants (astragalus
241 polysaccharide and propolis) with FCA used in pentavalent vaccines. In that study, FCA
242 outcompeted the other adjuvants although the natural adjuvants induced some
243 immunostimulant activities.

244 It has generally been difficult to develop effective vaccines against *A.*
245 *hydrophila* most probably because of the high degree of antigenic variation [17, 21, 22],
246 this is in contrast to vaccines against Gram-negative pathogens of salmonids like
247 *Aliivibrio salmonicida*, *Vibrio anguillarum*, *Yersinia ruckerii* and *A. salmonicida* –

where vaccines show up to 100% efficiency. Recently, a vaccine against *A. hydrophila* giving protection in rainbow trout was prepared [20]. LaPatra and co-workers developed a new challenge model in rainbow trout with *A. hydrophila* by injection into the dorsal sinus to determine the efficacy of a bacterial lysate. The vaccine was shown to give protection after i.p administration, and this protection could be potentiated in the presence of FCA [20]. Also, fish that survived an *A. hydrophila* challenge were very resistant to re-infection.

Flavobacterium psychrophilum is a widespread Gram-negative pathogen in freshwater causing rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) [23]. In addition to rainbow trout, coho salmon is the most susceptible species together with other non-salmonid species that are also affected. Injection of a low molecular weight fraction emulsified in FCA resulted in an enhanced level of protection for rainbow trout [23].

Flavobacterium columnare is a Gram-negative bacterium responsible for columnaris disease. The disease was first described in 1917 in several warm water fish species from the Mississippi river, and since has been isolated from freshwater fish species worldwide [24]. Specific antibodies were found in tilapia plasma and mucus following i.p. injection of formalin-killed sonicated or whole cells of *F. columnare* in FCA within 2 weeks. After a secondary immunisation, the antibody response increased and at 10 weeks post-immunisation the titre remained elevated. Also, antibodies were observed in cutaneous mucus in fish i.p. immunised with formalin-killed sonicated cells (ultrasound disrupted cells) in FCA 6 and 8 weeks post-immunisation [24].

3.1.2. Freund´s incomplete adjuvant

Because of its high toxicity, the use of FCA has been widely replaced by Freund´s incomplete adjuvant (FIA) that lacks the mycobacterial components of the emulsion, being therefore just a W/O emulsion. This adjuvant is still highly effective in vaccination with a significant reduction of toxicity, however, some important side effects are still present, effects very well detailed for Atlantic cod (*Gadus morhua*) in a very recent paper [25].

Edwardsiella tarda is a Gram negative intracellular bacterium that can infect both marine and freshwater fish, including Japanese flounder. In order to develop effective vaccines against this pathogen, fish were i.p. injected with a vaccine containing a major antigenic protein of *E. tarda* in the absence or presence of FIA [26]. Protection against experimental challenge achieved by the vaccine without adjuvant resulted in a relative per cent survival (RPS) of 34% that was increased to 81% in the presence of FIA. Moreover, vaccination with the oil-adjuvanted antigen stimulated the expression of a series of genes like complement component 3 (C3), MHC class I and MHC class II, CD8α, CD40, Mx, interferon γ (IFN-γ), tumour necrosis factor α (TNF-α) and interleukin 6 (IL-6), whereas vaccination with the antigen alone resulted in increased expression of just IgM, MHC class I and class II, and Mx [26].

Nocardia seriolae is a Gram-positive acid-fast bacterium that causes nocardiosis in cultured marine and freshwater fish in Taiwan, Japan and China. Although the disease results in considerable economic loss, no commercial vaccines are available. Very recently, an oil-adjuvanted vaccine was developed and tested on protection against challenge with a virulent strain [27]. Formalin-inactivated whole cell antigen was used as a vaccine with or without FIA, however, and even though antibody levels increased, no protective effects were found.

296 Another Gram-positive bacterium that causes disease (lactococcosis) and
297 mortality in rainbow trout is *Lactococcus garvieae*. Recently a vaccine was prepared
298 based on formalin inactivated bacterin or bacterin together with FIA. Fish were given
299 i.p. injections and challenged by exposure to virulent bacteria 30, 75, and 125 days after
300 vaccination [28]. A hundred and twenty five days after vaccination the RPS in fish
301 vaccinated with bacterin only was 54% and whereas it was 85% in fish vaccinated with
302 bacterin together with FIA.

303 *Tenacibaculum maritimum* is a marine bacterium that causes flexibacteriosis
304 worldwide. In Australia (Tasmania), Atlantic salmon (*Salmo salar*) and rainbow trout
305 are the most heavily affected species, and due to the lack of vaccines, so far the disease
306 has been treated with trimethoprim and oxytetracycline with the subsequent negative
307 impact on the environment [29]. Salmon injected with formalin inactivated bacteria
308 mixed with FIA provided protection against challenge with *T. maritimum* while the
309 vaccine without the adjuvant could not provide sufficient protection against a moderate
310 challenge of *T. maritimum*.

311 Infection with fungi oomycetes such as *Aphanomyces invadans* may cause heavy
312 mortalities of fresh water and estuarine fish species as a result of granulomatous
313 inflammation. In catla (*Catla catla* Hamilton), fungal extract combined with FIA
314 showed to increase both the survival rate during experimental challenge with *A.*
315 *invadans* and the antibody response compared to non-adjuvanted vaccines [30].

316

317 *3.1.3. Montanide*

318 Mineral oil adjuvants registered under the trademark of Montanide have been
319 optimised in order to improve efficacy and stability of vaccine formulations and to

reduce side effects. These adjuvants are based on either mineral oil, non-mineral oil or a mixture of both, as well as those made from specific surfactant chemistry using mannitol oleate and may be used to manufacture different type of emulsions, W/O, O/W or W/O/W, for use in both mammals and fish [31, 32].

Philasterides dicentrarchi is a scuticocilate parasite that causes mortalities and significant economic losses in cultured turbot [33]. An important attempt to optimise a vaccine against this parasite was performed on the basis of antigenic dose, concentration of inactivating agent (formalin) and proportion of the adjuvant Montanide ISA763A (W/O, non-mineral oil) in the emulsion. The results of the study showed that a high concentration of antigen, 0.2 % formalin and 50 % adjuvant generated the longest time of survival after challenge 30 days after the second injection, and the highest levels of antibodies in the vaccinated fish [33].

Pseudomonas plecoglossicida is a bacterium causing bacterial hemorrhagic ascites of cultured ayu (*Plecoglossus altivelis*). To develop a vaccine against the disease, formalin-killed *P. plecoglossicida* bacterin was emulsified with Montanide and injected i.p. The fish were challenged with an i.p injection of virulent *P. plecoglossicida* 22 and 52 days after vaccination [34]. The RPS of vaccinated fish was 17-58% without adjuvant, 57-92% with Montanide ISA711 and 65-86% with Montanide ISA763A. Another study on the same disease and adjuvant (Montanide ISA 763A) concluded that there is a good correlation between antibody levels and protection against disease in a challenge test [35].

To study the efficacy of different adjuvants in Atlantic halibut (*Hippoglossus hippoglossus*), fish were injected i.p. with a model vaccine of human gamma globulin with either FCA or Montanide ISA711 as adjuvants [36]. Antibody responses and

intraperitoneal adhesions were examined every month for up to 12 months. FCA produced the highest and fastest antibody response, since in the group injected with the Montanide adjuvant only 4 of 47 fish reached a titre of 1:1000 (month 6) compared to 27 of 48 fish in the FCA group (after 2 months), however, FCA also induced the fastest intraperitoneal adhesions [36].

In a very recent study in carp (*Cyprinus carpio*), a recombinant S-layer protein of *A. hydrophila* was used to assess the ability to protect fish against six virulent isolates of *A. hydrophila*. The recombinant S-layer protein of *A. hydrophila* was produced, diluted in phosphate buffered saline and mixed with a Montanide adjuvant at a ratio of 30:70. Common carp were i.p. injected with the emulsion, and after 35 days the fish were challenged with six different isolates of *A. hydrophila* [37]. The RPS values varied between the different challenge isolates (40-75%), but they suggested that the S-layer protein together with Montanide adjuvant is a good candidate for an efficacious vaccine.

Furthermore, Montanide ISA-763 has also been used as an adjuvant in experimental bivalent vaccine for *L. garvieae* and *A. hydrophila* with high degree of efficacy in rainbow trout [38].

3.1.4. Other mineral oil adjuvants

Moritella viscosa is the causative agent of winter ulcers in farmed fish like Atlantic salmon and Atlantic cod. Vaccination of Atlantic salmon against *M. viscosa* is performed with oil-adjuvanted polyvalent injection vaccines based on formalin-inactivated bacterial cultures, using an AJ-oil (Alpha Ject 5200) used in some vaccines commercialised by Pharmaq [39]. However, a multivalent commercial salmon vaccine containing *M. viscosa* as one of five bacteria mixed in a mineral oil adjuvant (Alpha Ject

5200) did not protect turbot against challenge [40], whereas moderate intra-abdominal adhesions were detected in vaccinated fish.

Other commercial oil-adjuvanted vaccines have been shown to give protection in Atlantic salmon against bacterial diseases like vibriosis, coldwater vibriosis and furunculosis for a long time. However, side effects and retardation in growth have been clearly demonstrated [41, 42]. Mutoloki and co-workers investigated the intraperitoneal lesions induced by an oil-adjuvanted vaccine against infection with *A. salmonicida* and *M. viscosa* in Atlantic salmon [43]. The cellular composition was typical of granulomas containing large macrophages, eosinophilic granular cells, lymphocytes and multinucleated cells.

Oil-adjuvanted vaccines are also used to control sea bass (*Dicentrarchus labrax*) against bacterial diseases like vibriosis and pasteurellosis. Sea bass is one of the most explored fish species in the Mediterranean area, and suffers from infection by *V. anguillarum* and *Photobacterium damsela* subsp. *piscicida*. Oil-adjuvanted vaccines against these diseases have been prepared and injected i.p., but despite their effectiveness, granulomatous peritonitis was also recognised [44].

The major bacterial disease of farmed Atlantic cod is classical vibriosis [45]. Cod vaccinated by injection with mineral oil adjuvanted vaccines against both *V. anguillarum* and atypical *A. salmonicida* were very well protected against homologous challenges [46]. In this model, even without adjuvant the fish were protected against *V. anguillarum*, but not against atypical *A. salmonicida* challenge.

3.2. Nano/ microparticles as adjuvants

Microparticles offer a promising option to oil emulsions, and their beneficial use as carriers for vaccine delivery has been widely discussed [47]. An association or/and encapsulation of antigen(s) with/in microparticles can be achieved by covalent linkage or physical entrapment. Compared to the latter technique, where the antigen is non-covalently and physically incorporated in the interior of the microparticle, covalent coupling offers distinct advantages: fewer amount of antigen is required; processing and presentation by antigen-presenting cells is more efficient; a higher stability during storage is obtained and any excess of (valuable) material can easily be regained. With the use of microparticles, a very low dose of antigen can give rise to an optimal humoral response.

The structure and the properties of microparticles may change markedly with slight alterations in production conditions, but nanoparticles can be prepared in a physico-chemically reproducible manner within narrow size limits. For this reason, adjuvants on the basis of these submicron polymeric particles were developed and have also been suggested for use as potent adjuvants in mammalian systems [48].

3.2.1. PLGA particles

Encapsulation of vaccines in biocompatible and biodegradable Poly-(lactide-co-glycolide) (PLGA) polymers has been studied for over twenty years. Antigen is released from the microspheres by diffusion through matrix pores and by matrix degradation. Biodegradation rates can be regulated by alterations in polymer composition and

412 molecular weights. In addition, there is often instant release of surface associated
413 antigens that may be beneficial to aid a rapid response.

414 So far, a few studies have been carried out on fish with regard to uptake and
415 degradation of PLGA particles and the immune response obtained. For the most part,
416 these studies have been on oral administration [49-53]. A recent article appeared on
417 parenteral immunisation of Indian major carp, rohu (*Labeo rohita*) with PLGA
418 encapsulated antigen [54]. Outer membrane proteins (OMP) of *A. hydrophila* were
419 encapsulated in PLGA microparticles and mixed with FIA in an emulsion or
420 administered alone by i.p. injection in rohu. Twenty-one and 42 days after
421 immunisation, the antibody titres were significantly higher in the PLGA-encapsulated
422 antigen group containing FIA [54].

423 A dose-dependent transient increase of antibody response following i.p injection
424 of PLGA particles containing human gamma globulin (HGG) has been shown by
425 Fredriksen and Grip [55] where it was shown that microparticle carriers were superior
426 compared to nanoparticles. Furthermore, when the formulation of PLGA entrapped
427 HGG was performed with ß-glucan or oil, it resulted in a continuous increase of
428 antibodies over time (over 120 days).

429 Oral vaccines encapsulated in PLGA have been also used in Japanese flounder
430 [51, 53] and salmonids like rainbow trout [50, 52, 56] or Atlantic salmon [49]. In the
431 case of Japanese flounder, a plasmid encoding the major capsid protein of lymphocystis
432 disease virus (LCDV) was constructed and encapsulated in PLGA. Controls were naked
433 plasmid vaccine and blank PLGA particles [53]. The fish were orally intubated, and 28
434 days post vaccination the fish were challenged by intramuscular injection with LCDV.
435 Vaccine-effects were evaluated by observing the presence of lymphocystis nodules. The

436 cumulative percentage of Japanese flounder with nodules after challenge was greatly
437 reduced in the group receiving the plasmid coding for the LCDV protein in PLGA
438 particles in the period of 15 to 120 days post-immunisation [53]. In addition, the levels
439 of antibody in sera of fish vaccinated with PLGA microcapsules increased for up to nine
440 weeks; although from this point it started to decrease [51].

441 In rainbow trout, oral vaccination (as a feed additive) against lactococcosis was
442 attempted with antigens encapsulated in PLGA particles [52]. RPS of the PLGA-
443 vaccine amounted to 63 % and booster vaccination with oral administration of the
444 PLGA-vaccine gave a RPS of more than 60 % 120 days after the first vaccination. Also
445 in rainbow trout, human gamma globulin (HGG) was microencapsulated in PLGA [50].
446 Specific antibodies were detected in the intestinal mucus of fish that were administered
447 with the microencapsulated antigen after boosting with soluble HGG, but not in fish that
448 were primed with the soluble antigen. The fate of orally administered HGG in Atlantic
449 salmon was determined, demonstrating that fifteen minutes after administration, the
450 HGG-PLGA was found in the intestine resembling the observation for free HGG [49].
451 The results from this study indicate that orally delivered HGG-PLGA had higher levels
452 and greater persistence of HGG systemically than free HGG.

453 Finally, feeding of rainbow trout with feed containing plasmid DNA encoding
454 IHNV G protein induced slightly higher amount of neutralising antibodies against
455 IHNV but no increased survival after experimental challenge with IHNV [56].

456

457 *3.2.2. ISCOMs*

458 Immune-stimulating complexes (ISCOMs) were conceived to co-formulate antigen
459 and adjuvant in a particle [57]. ISCOMs represent an interesting approach to stimulation

460 of the humoral and cell-mediated immune response towards amphipathic antigens. They
461 are relatively stable but non-covalently-bound complex of approximately 40 nm
462 diameter of saponin adjuvant Quil-A (saponin extracted from the cortex of the South
463 American tree *Quillaja saponaria molina*), cholesterol and amphipathic antigen in a
464 molar ratio of approximately 1:1:1. ISCOMs produced through the patented Matrix^{TM}
465 technology by Isconova have been widely studied in combination with different
466 veterinary vaccines, and are currently incorporated in a number of commercialized
467 animal vaccines. At this moment, Pharmaq is studying the introduction of these
468 adjuvants in commercialised fish vaccines.
469
470
471 **4. Signal 2 facilitators and TLR ligands as adjuvants or immunostimulants**
472
473 A large number of adjuvants that have been investigated do not directly affect
474 the concentration and distribution of antigen between injection site and presentation site
475 (this has not been established in fish yet). This category of vaccine adjuvant has
476 dominated the literature on vaccine research in the last decade, and comprises the
477 category of signal 2 facilitators, which include stranger and danger molecules, as well as
478 inflammatory cytokines.

479 A number of so-called toll-like receptors (TLR) ligands (agonists) may induce
480 strong innate responses that may be decisive for the outcome of acquired responses.
481 Teleost fish species may possess close to twice the number of different TLR compared
482 to mammalian species presumably due to an ancient genome duplication event. Many
483 similarities between mammalian and fish with respect to intracellular and downstream
484 signaling events exist, but there are dissimilarities that warrant focus. In this issue, a

485 detailed review authored by Aoki and Robertsen has been included, giving an excellent
486 overview of the current knowledge on fish TLR (technical editor: Check whether this
487 review is included in the special issue, and give reference). Another up-to-date review
488 on immune relevant genes including TLR-like receptors in fish is also authored by Zhu
489 *et al.* [58]. In general, those TLRs that, after ligand binding, induce the production of
490 IL-12 favour a Th1 response (TLR 3, 4, 5, 7, 8, 9 and 11) and in addition, the activation
491 of these TLRs may induce cross-presentation of antigens facilitating a cytotoxic T cell
492 response under certain conditions [59]. It should be mentioned that ligand binding to
493 TLRs 3 and 4, 7 and 9 may also induce type I IFN responses via interferon regulating
494 factors. Within this group of signal 2 facilitators, we have also included alum, as it has
495 been recently discovered that this adjuvant directly interacts with dendritic cells in a
496 similar way to that of danger signals [60].
497
498 *4.1. Aluminium containing adjuvants*
499 The adjuvant property of aluminium salts was discovered in 1926 [61].
500 Aluminium compounds, especially aluminium phosphate and aluminium hydroxide, are
501 some of the few adjuvants that have been allowed and considered safe to use in human
502 vaccines. Aluminium adjuvants have been shown to induce Th2 responses almost
503 exclusively [26], thus they have been used as adjuvants with great success, being
504 particularly effective at promoting protective humoral immunity. However, alum is not
505 optimally effective for diseases where cell-mediated immunity is required for
506 protection. It was believed that alum activates NLRP3 inflammasome and induces
507 necrotic cell deaths that release the danger signal uric acid [62]. However, very recently,
508 it has been discovered that being a crystal, alum binds dendritic cell plasma membrane
509 lipids with substantial force, independent of inflammasome and membrane proteins

510 [60]. The subsequent lipid sorting activates an abortive phagocytic response that leads
511 to antigen uptake. Such activated dendritic cells, without further association with alum,
512 show high affinity and stable binding with $CD4^+$ T cells via the adhesion molecules
513 intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated
514 antigen-1 (LFA-1). Only a few studies have been performed with aluminium adjuvants
515 in the optimization of fish vaccines.

516 Fifteen years ago a vaccine against *A. salmonicida* mixed with potassium
517 aluminium sulphate (alum) as an adjuvant was tested in Atlantic salmon [63]. Alum
518 appeared to enhance the protection against challenge, but not at a statistically significant
519 level. In another study, an *Escherichia coli* mutant was used for vaccination against
520 *Edwardsiella ictaluri*-induced enteric septicaemia of catfish (*Ictalurus punctatus*).
521 Killed *E. coli* bacteria with or without alum were administered i.p to catfish and the fish
522 were challenged with virulent *E. ictaluri* bacteria [64]. Fish given *E. coli* in alum
523 showed an enhanced survival (92 %) compared with the fish for which *E. coli* was
524 administered alone (54%) or fish given saline (56 %).

525 Recently, an aluminium hydroxide adjuvanted *E. tarda* vaccine was prepared
526 and injected i.p in Japanese flounder. The RPS was found to be 69 % [26] while
527 immunisation with the antigen alone followed by an experimental challenge gave a RPS
528 of 34, however, the FIA coupled vaccine showed a RPS of 81%.

529 Another experiment has been recently carried out by Fan *et al.* [65], in which
530 formalin-inactivated reddish body iridovirus (TRBIV) were mixed with alum and either
531 injected or bath administered twice in turbot. The resulting RPS calculated was 83.3%
532 and 90.5%, respectively.

533

534 *4.2. ß-glucans – ligands for dectin-1*

535 ß-glucans are known to stimulate the non-specific immune response of both
536 mammals and fish where dectin-1 may be involved [66, 67]. To obtain protective effects
537 against diseases the glucan is injected i.p., and there seems to be a dosage-dependent
538 and short-lived protection. In addition, there are some reports on the adjuvant effect of
539 ß-glucans [41, 42, 68-75].

540 DeBaulney and co-workers prepared an oral vaccine against vibriosis for use in
541 turbot, and after feeding the vaccine for 5 days the fish were challenged 28 days
542 thereafter. Fish given the vaccine alone resulted in a RPS of 52 %, while a combination
543 of the vaccine and the ß-glucan gave a RPS on 61 %, higher protection levels but not
544 statistically different from the vaccine alone [71]. In 1998, an attempt to establish
545 immunisation protocols to obtain the highest immune response against *V. damsela* was
546 performed in Spain [72]. In this study they i.p. injected the O-antigen of *V. damsela* in
547 combination with ß-glucan. As a correlate to vaccine efficacy, the phagocytic index of
548 head kidney macrophages was evaluated. The obtained results were as follows: the
549 enhancement of the phagocytic index lasted longer in fish injected with ß-glucan at the
550 same time or after being injected with the antigen when compared with fish injected
551 with ß-glucan before the antigen. Similar results were obtained with regard to antibody
552 titres [72].

553 Yeast glucan (mainly a ß-1,3-D glucan) was included in a furunculosis vaccine
554 that consisted in a formalin-killed culture of *A. salmonicida* and *V. salmonicida* [70].
555 The vaccine, either with or without ß-glucan, was injected i.p. and salmon challenged 3-
556 46 weeks after vaccination. Vaccines supplemented with ß-glucan induced significantly
557 higher protection against furunculosis than vaccines without this adjuvant [70], but ß-
558 glucan alone did not result in protection after 11 weeks. In another study, ß-glucan-

559 adjuvanted vaccines against furunculosis seemed to give protection at an early time-
560 point after vaccination (6 weeks), but no protection was seen after 3 and 6 months [41].
561 As a side effect, the average weight of the ß-glucan-adjuvanted group was significantly
562 lower compared to the controls, but the weight of fish given oil-adjuvant was also
563 significantly lower than the ß-glucan-adjuvanted group [42]. In a further study
564 performed in coho salmon, Nikl *et al*. evaluated the potentiating effect of seven
565 substances on the protection after vaccination with formalin-treated *A. salmonicida*
566 bacterin [68]. Statistically significant improvement in survival over the group receiving
567 bacterin alone was noted in fish groups that also received ß-glucans like Vitastim-Taito
568 and lentinan. However, agglutinin levels were significantly elevated in all cases where
569 the bacterin was injected, and no significant elevation in agglutinin titer occurred as a
570 result of combining an immunostimulant with the bacterin [68].

571 Catla is one of the major Indian carp species often affected with *A. hydrophila*,
572 thus a formalin-inactivated *A. hydrophila* vaccine was developed and protection was
573 studied in the absence and presence of a ß-glucan adjuvant [74]. A reduction in
574 mortality was found in the presence of ß-glucan compared to the vaccine itself, although
575 the differences were not statistically significant (RPS of 67.7 % and 58.0% with and
576 without the adjuvant, respectively). In carp, a vaccine against *A. hydrophila* showed a
577 higher antibody titer when ß-glucan was i.p. injected prior to vaccination, while bath
578 and oral administration of ß-glucan before vaccination did not result in enhanced
579 antibody response [75]. In a further study by Selvaraj and coworkers, carp were
580 vaccinated against *A. hydrophila* with LPS from a virulent strain of the bacterium in the
581 presence of different concentrations of ß-glucan and administered through various
582 routes such as i.p, oral or bath [76]. The RPS was significantly higher in i.p. injected
583 groups even at the lowest concentration of ß-glucan and fish given a mixture of LPS and

584 ß-glucan orally obtained a higher RPS compared to controls. The administration of the
585 LPS-glucan by bath did not result in increased survival, and antibodies were never
586 detected in fish vaccinated either orally or by bath. However, no possible analysis of the
587 contribution of β-glucan in the vaccine efficacy could be established because an obvious
588 control group in this study was missing, namely the protective effect of LPS without
589 adjuvant [76].

590 In another study, the i.p. injection of ß-glucan on days 1 and 3 followed by two
591 i.p. immunisations of *E. ictaluri* on days 7 and 14 performed in channel catfish resulted
592 in higher serum antibody levels relative to catfish receiving PBS instead of ß-glucan
593 before administration of *E. ictaluri* [69]. Serum antibody levels were determined on day
594 7 (day 21) after the last immunisation, reaching with ß-glucan antibody titers that were
595 typically two-fold higher than those of fish without ß-glucan.

596 In order to investigate possible treatments against *A. hydrophila* in blue gourami,
597 laminaran, a ß-1,3-D-glucan, was injected i.p. in the absence and presence of formalin-
598 killed *A. hydrophila* bacteria [77]. A single i.p. injection of 20 mg kg^{-1} laminaran alone
599 was sufficient to protect the fish against infection by a virulent strain of *A. hydrophila*
600 up until 29 days after injection in correlation with an increased phagocytic activity of
601 head kidney phagocytes. Despite this, the addition of 20 mg kg^{-1} laminaran to a
602 formalin-killed *A. hydrophila* did not significantly improve the protection [77].

603

604 *4.3. Saponins*

605 Saponins are natural glycosides of steroid or triterpene which have been widely
606 explored as adjuvants in different mammalian systems due to their capacity to stimulate
607 both Th1 and Th2 responses [78]. The most widely used saponins are Quil A

608 (component of ISCOMs) and their derivatives, however, due to their high cytotoxicity
609 and instability in aqueous phase, the use of different kinds of saponins is being
610 explored.

611 In Japanese flounder, formalin-killed *E. tarda* cells were administered to fish by
612 feeding in the absence or presence of curdlan or curdlan together with Quil A saponin.
613 Although the incorporation of curdlan gave higher survival rates, only the group in
614 which the vaccine was administered with both curdlan and Quil A showed significantly
615 better survival [73].

616

617 *4.4. Poly I:C – toll-like receptor 3 agonist*

618 Polyinosinic polycytidylic acid (poly I:C) is a double stranded
619 polyribonucleotide, that mimics a viral infection and therefore has been widely used to
620 induce a type I IFN in many species including fish [79-81]. IFNs are cytokines with a
621 major role in the early defence against viral infections, thus Poly I:C induces a non-
622 specific antiviral state after its binding to TLR3 and the subsequent activation of
623 intracellular signalling events. This non-specific antiviral activity of Poly I:C has been
624 recently tested in rainbow trout infected with infectious hematopoietic necrosis virus
625 (IHNV) [82]. Fish pre-injected with Poly I:C were protected against IHNV challenge 2
626 days later and IHNV-specific antibodies were detected in survivors. The survivors
627 showed a 100% survival rate following re-challenge with IHNV both 21 and 49 days
628 after the primary IHNV challenge [82], demonstrating that the fact that fish were at an
629 antiviral state during the encounter of a virus, gave them an important advantage for
630 posterior specific antibody production. A similar study was performed in the sevenband
631 grouper *Epinephelus septemfasciatus* in which fish were immunised against the

nodavirus red-spotted grouper nervous necrosis virus (RGNNV) [83]. Fish injected with 50 mg Poly I:C $fish^{-1}$ or more intramuscularly (i.m.) and challenged i.m. with RGNNV 2 days post-injection showed more than 90% survival rate. When surviving fish were re-challenged with RGNNV 3 weeks after the primary challenge, no mortalities were detected in the group that had been previously exposed to Poly I:C, since upon RGNNV challenge the antibodies against RGNNV were higher in these fish. All survivors that were re-challenged with RGNNV showed even higher levels of specific antibodies. In addition, the RGNNV titres in brain tissues of the survivors in the Poly I:C-RGNNV-RGNNV group were all under the detection limit [83]. Following up this work, this research group conducted a field trial exploring the vaccine efficacy of a RGNNV vaccine followed by Poly I:C injection. The Poly I:C-adjuvanted vaccine showed reasonable efficacy, but a one-shot Poly I:C injection in sevenband grouper 20 days after a natural RGNNV outbreak also induced a high survival rate (93.7%) compared to non-treated fish (9.8%) [84].

A prophylactic strategy using poly I:C was also used by Takami and co-workers in Japanese flounder experimentally infected with viral haemorrhagic septicaemia virus (VHSV) [85]. The survival rate in Japanese flounder after VHSV challenge following Poly I:C administration was 100%, while all untreated fish died within 9 days. Survival rates of the fish given a secondary challenge VHSV were 100% in the Poly I:C-VHSV group (Poly I:C-VHSV-VHSV group), while non-immunized fish showed a 0% survival.

654 *4.5. Lipopeptides*

655 Lipoproteins and lipopeptides have been found in a large number of
656 microorganisms, the most prominent being mycobacteria and mycoplasms. These
657 molecules have been found to exhibit both a strong innate (inflammatory) response and
658 a long-lasting adaptive immune response in mammals. Very few studies have been
659 performed on lipopeptides in fish. The adjuvant effect of polar glycopeptidolipids in
660 experimental vaccines against *A. salmonicida* was investigated [86]. Polar
661 glycopeptidolipids (pGPL-*Mc*) were extracted from *Mycobacterium chelonae*, which is
662 one of three mycobacteria species that are fish-pathogenic. At 12 weeks post
663 vaccination, the antibody response of fish given 0.25 mg kg^{-1} pGPL-*Mc* in combination
664 with an *A. salmonicida* bacterin was significantly higher than that induced by a non-
665 adjuvanted bacterin. Increased doses of pGPL-*Mc* suppressed the antibody response,
666 while no significant side effects were observed in the peritoneal cavity after use of this
667 adjuvant [86].

668

669 *4.6. Flagellin – toll-like receptor 5 agonist*

670 The structural protein of Gram-negative flagella is called flagellin. Flagellin is a
671 potent activator of a broad range of cell types within the innate and adaptive immune
672 system. Several studies have demonstrated the ability of flagellin as an adjuvant,
673 promoting cytokine production [87]. Flagellin is known to induce immune responses via
674 the TLR5 signalling resulting in a mixed Th1 and Th2 response, although it has also
675 been reported that inflammasomes containing NLRC4/IPAF may bind cytosolically
676 located flagellin [62]. During the last decade, the adjuvant effect of flagellin has widely
677 been studied in vertebrates and during the last couple of years also in fish [88-90]

678 Piscirickettsiosis is a severe disease reported in salmonids that has caused
679 especially great problems for the Chilean aquaculture industry. In 1989, the bacterium
680 *Piscirickettsia salmonis* was isolated from a moribund coho salmon and was found to be
681 the etiological agent of this disease. The pathogen is a Gram-negative obligate
682 intracellular bacterium. The disease has also been reported to affect Atlantic salmon,
683 rainbow trout and other farmed salmonid species [88]. A recombinant subunit vaccine
684 was developed in order to control the disease due to poor responses after treatment by
685 antibiotics. Three experimental formulations were prepared containing two or three
686 recombinant proteins of the bacterium, and the formulations were emulsified with one
687 volume of FIA [88]. The highest protective response was obtained with a vaccine
688 formulation containing the subunit of the flagellum and chaperonins Hsp60 and Hsp70
689 of *P. salmonis*, suggesting that the use of more than one recombinant protein antigen is
690 needed to obtain a good protective effect against this disease.

691 Jiao and co-workers have been studying different vaccine concepts against *E.*
692 *tarda* in the Japanese flounder to obtain effective protective formulations, based on both
693 recombinant proteins and DNA vaccine constructs [89, 90]. The most promising
694 vaccine concept was the one consisting in a chimeric DNA vaccine coding for the *E.*
695 *tarda* proteins Eta6 fused in-frame to FliC, the flagellin for *E. tarda*. Although they
696 found that *E. tarda* FliC induced low protective immunity by itself, it could function as
697 a molecular adjuvant and potentiate the specific immune response induced by the *E.*
698 *tarda* antigen Eta6. Fish immunised with pEta6 and FliC produced specific serum
699 antibodies and exhibited enhanced expression of genes that are involved in both innate
700 and adaptive immune responses (IL-1ß, IFN, Mx, CD8α, MHC-Iα, MHC-IIα, IgM)
701 [89, 90]. Such up regulation following immunisation with flagellin has also been
702 described by Hynes *et al.* [91], where TNF-α, IL-6, IL-8 and IL-1ß were significantly

703 up regulated compared to non-adjuvanted controls. In this study, however, there was no
704 induction of specific antibody response against flagellin or the model antigen *Limulus*
705 *polyhemus* hemolymph (LPH) in the Atlantic salmon.

706

707 *4.7. CpG – toll-like receptor 9 agonist*

708 Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) expressing
709 unmethylated CpG motifs trigger an immunostimulatory cascade that culminates in the
710 maturation, differentiation and proliferation of multiple immune cells, including B and
711 T lymphocytes, NK cells, monocytes, macrophages and dendritic cells. CpG motifs are
712 approximately 20 times less common in mammalian than microbial DNA, and therefore
713 are recognised as a danger signal by cells that express TLR9. In mammals, it has been
714 widely demonstrated that CpG ODNs function as adjuvants when co-administered with
715 vaccines, being able to both accelerate and magnify the immune response [92]. In fish,
716 although many studies have been carried out on the immunomodulatory effects of CpGs
717 [93-96], only few studies have focused on the adjuvant effect of these molecules.

718 Chinook salmon (*O. tshawytscha*) reared in the Pacific Northwest of the United
719 States often suffers from infection with *Renibacterium salmoninarum*, the causative
720 agent of bacterial kidney disease (BKD). The conclusion from a study in which whole
721 cell vaccines with or without CpG adjuvants were used, was that either the vaccine
722 alone or that with CpG provided protection against i.p. challenge with *R. salmoninarum*
723 [93]. However, a combination of a commercial *R. salmoninarum* vaccine (Renogen)
724 with a CpG adjuvant significantly reduced the level of bacterial antigens in the kidney
725 of naturally infected fish [93].

In a study in rainbow trout, four groups were i.m. injected with a commercially available, non-adjuvanted aqueous vaccine against furunculosis containing inactivated cultures of *A. salmonicida* (Aquavac Furovac 5) alone, or together with CpG ODN 1982, CpG ODNs 2133 or ODN2143. The fish were challenged with i.p. injection of a pathogenic strain of *A. salmonicida* 7 weeks after injection and the only group that showed a significantly lower mortality compared to those injected with Furovac alone (mortality of 52%) was the group injected with Furovac and the CpG ODN 2143 in which only a 21% of the fish died [94].

The protective effect of CpG motifs was also studied by Liu and co-workers in turbot and Japanese flounder [95, 96]. Sixteen CpG ODNs were synthesized and examined for the ability to inhibit bacterial dissemination in Japanese flounder blood. Four ODNs with the strongest inhibitory effects were selected and a plasmid pCN6 was constructed containing the sequences of the 4 selected ODNs. Japanese flounder were injected i.m. with plasmids pCN6 and pCN3 (control) and PBS. Four weeks post-vaccination the fish were challenged with *A. hydrophila* and mortality was monitored over a period of 20 days. Accumulated mortalities were 30%, 66.7% and 63.3% in pCN6-, pCN3, and PBS-immunised flounder, respectively [96]. Fish were also vaccinated as above and challenged with *E. tarda* 4 weeks after vaccination and the mortalities were 53.3, 90%, and 93.3% respectively. Therefore, the pCN6 plasmid provided a nonspecific protection against both *A. hydrophila* and *E. tarda* infections. This nonspecific protective effects have also been observed in fish parasitic infections since certain CpGs (e.g. CpG-ODN 1668 and CpG-ODN 2359) have also proved to have effects protecting fish against *Miamiensis avidus* [97]. Following on, a salmonid alphavirus (SAV) vaccine containing antigen plus CpG and Poly I:C as adjuvants induced a significant production of neutralizing antibodies and conferred some level of

751 protection – as evaluated by percentage of SAV positive fish compared to controls [98] .
752 The authors reported that the adjuvanted vaccines induced prominent IFN type I
753 expression – that is crucial in antiviral response.

754 To analyse the adjuvant effect of CpGs in turbot, fish were vaccinated with a
755 *Vibrio harveyi* recombinant subunit vaccine, DegQ, in combination with a CpG that had
756 been shown to provide anti-bacterial effects in the host species after injection. Fish were
757 vaccinated by i.p. injection including all the appropriate controls and twenty-eight days
758 after vaccination, the fish were challenged by a virulent strain of *V. harvey*, and
759 accumulated mortalities were recorded [95]. The only vaccine formulation that induced
760 a significant protection was DegQ in combination with this pCN5 CpG. The duration of
761 the adjuvant effect was found to last at least 50 days. 07/02/2013

762 One of the unique features of DNA vaccines is the ability to stimulate both
763 cellular and humoral immune responses through the administration of a bacterial
764 plasmid coding for a protective antigen [99]. Thus, these DNA vaccines possess
765 intrinsic immunostimulatory capacity due to the presence of CpG motifs in the bacterial
766 plasmid backbone. Therefore, the inclusion of additional CpG motifs in the vaccine
767 plasmid would provide us with an intrinsic adjuvant within the same construct, being an
768 easy method to increase the immunogenicity. In this sense, a recent work by Martinez-
769 Alonso *et al.* [100] explored the possibility of increasing the immunogenicity of a
770 VHSV DNA vaccine though the introduction of several copies (either two or four) of a
771 fragment containing multiple CpG sequences of known immunostimulatory effects into
772 the DNA vaccine. The addition of these CpG motifs significantly increased the titre of
773 neutralising antibodies in serum and increased the levels of transcription of several
774 immune genes such as Mx or MHC-I, demonstrating for the first time that additional
775 CpG motifs may also be used to increase the immunogenicity of these DNA vaccines.

776

777 *4.8. Cytokines*

778 In the past years, a great number of cytokine genes have been identified in many
779 fish species, however, and despite the fact that the use of cytokines as adjuvants has
780 been widely explored in mammals, not many studies have focused on the possible use
781 of cytokine genes as vaccine adjuvants in fish. This may be due to the fact that for the
782 majority of these molecules, many details concerning their immunological role are still
783 lacking, and until we know what immune processes they are regulating, their use would
784 be a mere trial and error process. In any case, some attempts to explore their potential
785 have been made in some fish species.

786 Interferon regulatory factors (IRFs) form a large family of transcription factors.
787 IRF-1 has been shown to have a role in cytokine signalling and host defence against
788 pathogens. For example, IRF-1 is up-regulated in response to virus infection in fish
789 cells, inducing an antiviral state [101]. In a recent study, the potential use of IRF-1 as a
790 vaccine adjuvant was investigated in Japanese flounder. The co-injection of IRF-1 with
791 a DNA vaccine encoding the major capsid protein (MCP) gene of red sea bream
792 iridovirus (RSIV) resulted in elevated serum neutralisation antibodies but was not
793 significantly different from that in the fish vaccinated with the DNA vaccine alone
794 [102]. Despite the moderate effect in protection, in this study, IRF-1 was responsible for
795 the up-regulation of antiviral substances like nitric oxide (NO), interferon ß (IFN ß) and
796 interferon inducible genes such as Mx.

797 Interleukin 8 (IL-8) is a CXC chemokine produced by many cell types in
798 mammals like macrophages, monocytes, epithelial cells, neutrophils and fibroblasts
799 upon infection, or stimulated by cytokines like IL-1ß and tumor necrosis factor a (TNF-

α). In mammals, chemokines have been widely used as adjuvants in vaccines against viral infections, since not only they attract more cells to the site of inflammation, but they also regulate the immune functions of the recruited cells. In fish, IL-8 has been characterised in rainbow trout among other species, and its chemo attractant properties established [103]. In this species, a vaccine plasmid coding for the glycoprotein gene of VHSV was co-injected with a plasmid coding for rainbow trout IL-8 to explore its potential adjuvant effect [104, 105]. When the plasmid coding of IL-8 (pIL-8+) was administered together with the VHSV vaccine, an increase of IL-1ß in the spleen was found together with a greater cellular infiltration at the site of inoculation. Furthermore, fish injected with pIL-8+ alone showed a significantly higher expression of TNF-α, IL-11, TGF-ß and IL-18 in the spleen [104]. In a further study, the transcription of different inducible CC chemokines were studied in rainbow trout in response to both the VHSV DNA vaccine and/or pIL8+, demonstrating that when IL-8 is used as an adjuvant, the expression of other chemokines such as CK5A, CK6, CK7 and CK5B is also modulated [105]. All these results showed that IL-8 was able to modulate the early immune response and could be a potent vaccine adjuvant in fish against viral infections.

Administration of IL-1ß-derived peptides to rainbow trout by i.p. injection induced reduced mortality of fish when exposed to VHSV after 2 days [106]. The peptides also induced leukocyte migration into the peritoneal cavity 1-3 days post-injection, however its possible use as adjuvant was not further explored. The role of IL-1ß as an adjuvant was investigated in carp after i.p. injection of killed *A. hydrophila* in the absence and presence of recombinant C-terminal peptide of carp IL-1ß. It was found that the agglutinating antibody titre was significantly higher in the fish injected with killed bacteria plus recombinant IL-1ß peptide compared with killed bacteria alone 3 weeks after vaccination [107].

6. Conclusive remarks and perspectives

The development of effective vaccines should be approached by combining the search for protective antigens together with the application of specific, and targeting, adjuvants that maximise the immunogenicity with a desired immune response. These vaccine-specific adjuvants may be able to trigger specific immunological processes, without producing a generalised response with strong side effects. However, an obvious consequence for the lack of detailed knowledge on vaccine potency and efficacy using novel adjuvants such as the TLR ligands or cytokines - is that the vaccine producers may use oil-adjuvants instead for simplicity reason. The oil adjuvants, being able to induce very strong and durable immune responses may "overshadow" significant protective mechanisms that have been overlooked up till now. Thus, the search for real molecular correlates of protection should be pursued with strong efforts. In future vaccine research, the immunostimulatory potential of a given substance followed by vaccine potency and efficacy studies should be unequivocally established in the context of vaccination. Only then, we will be able to convince the pharmaceutical industry to move from traditional adjuvants to more sophisticated adjuvants that specifically trigger adequate immune responses that may be optimised for a specific pathogen.

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Table 1. Adjuvants, central components, receptors/process and principal immunological responses elicited by licensed and experimental adjuvants mainly for human medicine. Adapted from Coffman *et al.* [57].

Adjuvant	Central immunostimulatory component(s)	PPR/Process	Principal immune response elicited
Alum	Aluminum salts	NLRP3 (?)	Ab, Th2 (+Th1 in humans)
MF59 and AS03	Squalene in water emulsions	Tissue inflammation	Ab, Th1 and Th2
AS04	MPL + Alum	TLR4 and NLRP3(?)	Ab and Th1
Adjuvants in experimental use or in late stage clinical development			
Poly I:C	Synthetic dsRNA		Ab, Th1, CTL
MPL, and in diff. formulations			Ab, Th1
Flagellin, flagellin-Ag fusion proteins	Recombinant flagellin from bacteria	TLR5	Ab, Th1 + Th2
Imiquimods	Imidazoquinoline derivatives	TLR7, TLR8 and both	Ab, Th1, CTL (when conjugated)
CpG, and in different formulations	Synthetic phosphorthioate-linked DNA oligonucleotides with optimized CpG motifs	TLR9	Ab, Th1, CTL (when conjugated)
ISCOMS	Saponins	Not defined	Ab, Th1 + Th2, CTL
IFA (and montanide formulations	Mineral or paraffin oil + surfactant	Not defined	Ab, TH1 + Th2
CFA	IFA + peptidoglycan, trehalose dimycolate	NLR, TLR?	Ab, Th1, Th17

Abbreviations and descriptions: MF59 (Novartis proprietary adjuvant MF59 containing squalene, polyoxyethylene sorbitan monooleate and sorbitan trioleate), AS03 (GlaxoSmithKline) contains squalene, DL-a-tocopherol, polysorbate), AS04 (Aluminum hydroxide and monophoshoryl lipid A (MPL), ISCOMs (immune stimulating complex; nanostructure of cholesterol, phospholipids and Quil-A saponis), IFA (incomplete Freund´s adjuvants). Ab: antibodies.

1193 Table 2. Adjuvants currently used in fish vaccines commercialised by the main fish
1194 vaccine manufacturers.

Company	Vaccine name	Pathogen	Adjuvant	Immunization route
PHARMAQ	Alpha Ject and Alpha Marine vaccines	Different bacterial and viral pathogens	Mineral oil	i.p.
	Alpha Dip		No adjuvant	Immersion
MSD Animal Health	AquaVac	A. salmonicida, *Y. ruckeri, Vibrio*	No adjuvant	i.p., immersion
	AquaVac FNMPlus	A. salmonicida	Montanide ISA711	i.p.
	Norvax Compact PD	Salmonid alphavirus (SAV1 and SAV3)	Montanide ISA763A	i.p.
	AquaVac ERM Oral	*Y. ruckeri, Vibrio*	No adjuvant	oral
Novartis	Birnagen Forte As	A. salmonicida and infectious pancreatic necrosis virus (IPNV)	Mineral oil (Drakeol 6VR)	i.p.
	Ermogen	*Y. ruckeri*	No adjuvant	Immersion
	Apex®-IHN	IHNV	No adjuvant	i.m.

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